

## Chapter 4

# Patch clamp techniques for single channel and whole-cell recording

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### 1. Introduction

The *patch clamp* technique was first used by Neher and Sakmann (1976) to resolve currents through single acetylcholine-activated channels in cell-attached patches of membrane of frog skeletal muscle. The method they used (described by Neher, Sakmann & Steinbach, 1978) and subsequent refinements (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) have led to techniques for high resolution recording of current in *excised membrane patches* in addition to those that remain *cell-attached*. Single channel recording yields information about unitary conductance and kinetic behaviour of ionic channels already partly investigated by classical voltage clamping and by noise analysis; it is also leading to the discovery of new classes of ion channel. Together with the method of whole-cell recording, which permits the application of voltage clamping to cells that are too small for microelectrode methods, patch clamp techniques also permit investigations of the physiological role of ionic channels in cells otherwise inaccessible to voltage clamp and to cells that are not electrically excitable.

### 2. Method for patch clamp recording

The principle of the method is to isolate a patch of membrane electrically from the external solution and to record current flowing into the patch. This is achieved by pressing a fire-polished glass pipette, which has been filled with a suitable electrolyte solution, against the surface of a cell and applying light suction. Providing both glass pipette and cell membrane are clean, a seal whose electrical resistance is more than 10 G $\Omega$  is formed. Under such conditions, the glass pipette and the cell membrane will be less than 1 nm apart.

A high seal resistance is needed for two reasons. First, the higher the seal

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resistance, the more complete is the electrical isolation of the membrane patch. Secondly, a high seal resistance reduces the current noise of the recording, permitting good time resolution of single channel currents, currents whose amplitude is in the order of 1 pA.

Fig. 1 illustrates an equivalent circuit for the recording set-up. The fraction of the current flowing through the patch that will be collected by the recording pipette is

$$R_{\text{seal}} / (R_{\text{seal}} + R_{\text{pipette}})$$

where  $R_{\text{seal}}$  is the seal and  $R_{\text{pipette}}$  the pipette resistance.

As Fig. 1 also illustrates, it is necessary that the patch from which recordings are made be small in area, compared with the area of membrane of the cell as a whole. In terms of Fig. 1, it is necessary that  $R_{\text{cell}} \ll R_{\text{patch}}$  if single channel currents are not to alter the membrane potential of the cell significantly.

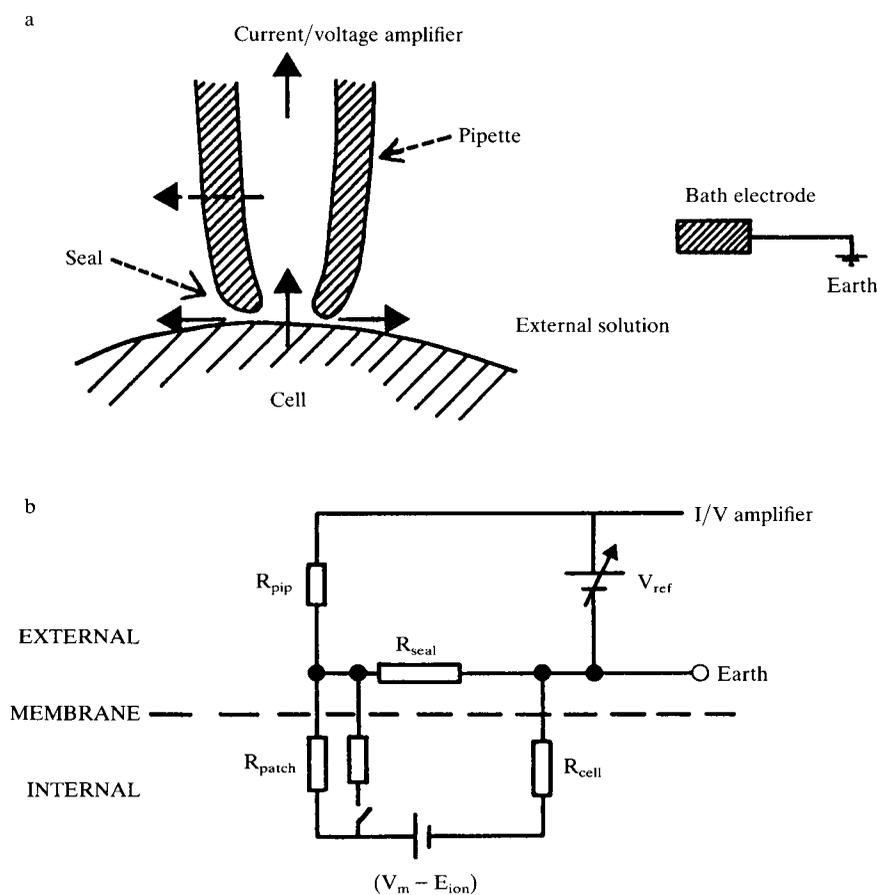


Fig. 1. The relation of pipette to cell and the equivalent electrical circuit during patch clamp recording. The opening of an ion channel is represented as the closing of the switch in Fig. 1b. The pipette resistance and seal resistance are in series between the amplifier and earth (the external solution) and the patch and cell resistances are in parallel with the seal resistance in this path.

The background noise level is also minimized by a high seal resistance. The variance of the current noise (in  $A^2$ ) through a resistor ( $R, \Omega$ ) is related to the Johnson voltage noise due to the resistance, being given by:

$$s_i^2 = 4kTf_c/R$$

where  $k$  is Boltzmann's constant,  $T$  is temperature ( $^{\circ}$ Kelvin), and  $f_c$  is the bandwidth (Hz) i.e. the low pass filter setting. Thus, for a  $10\text{ G}\Omega$  resistor at  $20^{\circ}\text{C}$ , the standard deviation of the current noise at  $1\text{ kHz}$  will be  $0.04\text{ pA}$ , but for a resistor of  $100\text{ M}\Omega$  it will be  $0.4\text{ pA}$ . In the recording situation used in patch clamp (Fig. 1), resistor current noise will depend on all the resistive paths to ground from the amplifier input, decreasing as resistance increases. Since the patch resistance is high (approximately  $100\text{ G}\Omega$  or more), the low seal resistance predominates and will result in noise that will prevent good resolution of currents smaller than 4 or 5 pA. Such was the situation in the earliest patch clamp experiments, where the seal resistance was less than  $100\text{ M}\Omega$ . In spite of this difficulty, information was obtained about acetylcholine-activated channels (Neher & Sakmann, 1976), about the blocking effects of local anaesthetics on such channels (Neher & Steinbach, 1978), and about glutamate-activated channels of insect muscle (Patlak, Gratton & Usherwood, 1979; Cull-Candy, Miledi & Parker, 1981). The achievement of 'gigaseals' ( $>1\text{ G}\Omega$ , Neher, 1981; Hamill et al. 1981), however, radically improved the quality of recording and made it possible to study channels of lower unitary conductance.

Such seals are generally achieved in an all-or-nothing fashion and result in a dramatic improvement in signal-to-noise ratio. The conditions that appear to be required for the formation of a gigaseal are the following.

First, the surface membrane of the cell used must be clean and free of extracellular matrix and connective tissue. Cells in tissue culture are often preferred; adult cells generally must be cleaned enzymatically or mechanically.

Secondly, solutions should be free of dust and of macromolecules such as the components of serum in tissue culture media. Solutions are filtered using  $0.2\text{ }\mu\text{m}$  filters (of the detergent-free type). Cell cultures are washed several times to remove serum.

Thirdly, the pipette tip is clean, often by fire-polishing.

Fourthly, during the period just prior to seal formation, a small positive pressure is applied to the pipette to generate an outflow of solution from the pipette tip and so keep it free of debris.

### *Loose-patch clamp*

In certain situations, however, a low seal resistance can be an advantage. Such a method is the so-called 'loose-patch' clamp (Almers, Stanfield & Stühmer, 1983), developed to allow measurement of the distribution of ionic channels over cell membranes and, making use of the low resistance seal and the resulting gap that will exist between membrane and recording pipette, to permit investigation of the lateral mobility of ionic channels. Here large diameter (approximately  $10\text{ }\mu\text{m}$ ) pipettes are used to collect current from many ionic channels at the same time. Currents that are

collected by the pipette must be multiplied by a 'seal factor' ( $\{R_{\text{seal}}+R_{\text{pipette}}\}/R_{\text{seal}}$ ) to correct for the fraction of the membrane current that flows to ground through the resistance of the seal.

The method has been used to investigate sodium channels of skeletal muscle, channels that are activated by stepping membrane potential. Changing the voltage of the inside of the pipette to achieve this change in membrane potential will result in large currents flowing through the resistance of the seal to the grounded bath solution. This leakage current must be subtracted. Additionally, the voltage applied as  $V_{\text{ref}}$  (in Fig. 1) must be bigger than the desired change in membrane potential by the seal factor ( $\{R_{\text{seal}}+R_{\text{pipette}}\}/R_{\text{seal}}$ ) to correct for the division that occurs between pipette and seal resistances. These corrections can be applied by a mixture of analogue and digital means. If a single pipette is used, no correction can be made for an additional source of error, activation at an uncontrolled voltage of the ionic channels in the membrane under the rim of the pipette, where the seal is formed. Concentric, double-barrelled pipettes have been used to remove this source of error (Roberts & Almers, 1984).

### *Gigaseal patch clamp*

Cell-attached and excised patches; whole-cell recording.

Fig. 2 summarizes the main modes of gigaseal recording. Much work is done using patches in the cell-attached mode, but the resting potential of the cell is not known and neither intra- nor extracellular ionic concentrations can be changed easily. For these reasons, it is sometimes essential to work using a cell-free mode, with excised or ripped-off patches. There are two kinds:

Inside-out - made by pulling the membrane patch off the cell into the bath solution.

Outside-out - made by applying suction to destroy the membrane isolated by the patch pipette and then pulling the pipette away from the cell. The membrane should reseal to give a patch of membrane whose intracellular face is in contact with the pipette solution.

Whole-cell recording is achieved by destroying the membrane patch using suction so that the cell, whose interior then comes into contact with the solution in the pipette, may be voltage or current-clamped. The cell contents equilibrate over time with the solution within the pipette (Fenwick, Marty & Neher, 1982). Further details of these modes are given below.

### *Giant patches and measurement of pump currents*

A procedure has been described for making gigaseals with large diameter pipettes (10-25  $\mu\text{m}$ ) and has been used to study electrogenic pump and exchange transport where the unitary currents are small (Hilgemann 1989, 1990; Collins, Somlyo & Hilgemann, 1993). It uses one of a variety of lipophilic 'glues' (eg. 40/60 w/w Parafilm/mineral oil,  $\alpha$ -tocopherol) to improve seal resistance and produces large areas of electrically isolated membrane. Currents generated by ion exchange processes such as the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, the sodium pump, and many exchangers driven by the electrochemical gradient on  $\text{Na}^+$ , are too small to resolve as unitary

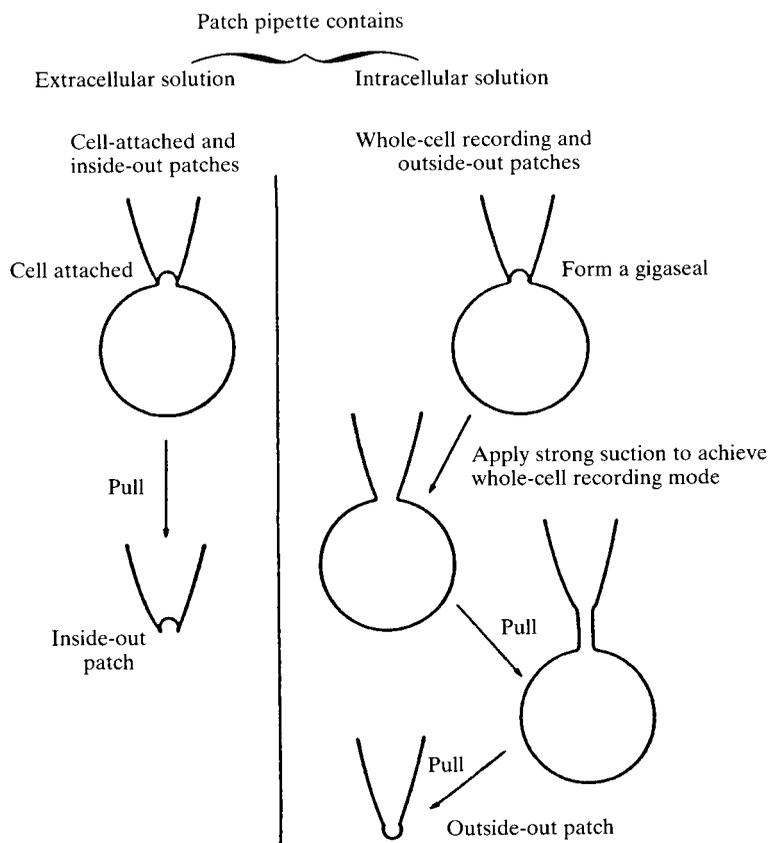


Fig. 2. Diagram illustrating the methods of making cell-attached and inside-out patches (left hand panel) and whole cell and outside-out patches.

currents and in normal size patches are not present in sufficient number to generate a measurable current. In large patches, currents due to exchangers and pumps are well resolved. They are used specifically in the inside-out configuration to permit changes at the cytosolic surface that modify the rate and characteristics of transport measured as the pipette current.

### 3. Instrumentation of single channel recording

A diagram of the essential features of the amplifier used in the headstage of a patch clamp is shown in Fig. 3. Essentially, this amplifier is a current-to-voltage converter which has a high gain, owing to the large feedback resistor  $R_f$ , and which is arranged so that the potential inside the pipette,  $V_{ref}$ , may either be held at a steady level or changed in a step-wise fashion. A description of the patch amplifier is given in Chapter 16, but the following points may be considered here.

- (i) The input of the amplifier has JFET transistors of low leakage current and noise

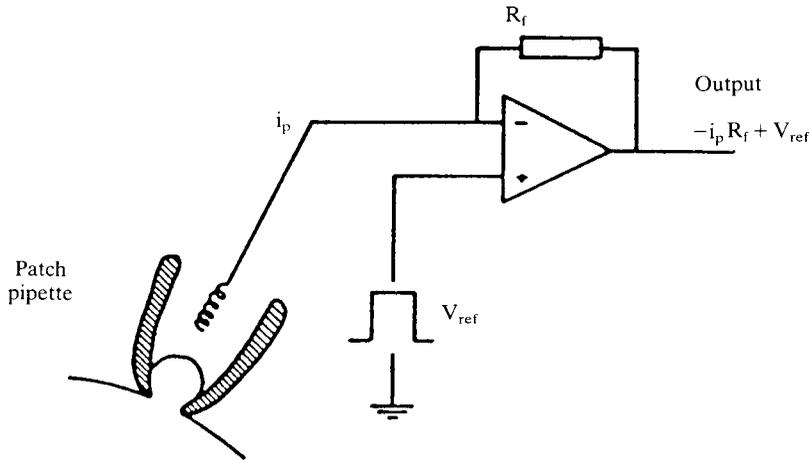


Fig. 3. Schematic diagram of the headstage current/voltage amplifier. The gain ( $V_o/i_p$ , mV/pA) is set by the feedback resistor  $R_f$ ,

$$V_o = -R_f \cdot i_p + V_{ref}$$

$V_{ref}$  is composed of the sum of  $V_{hold}$ ,  $V_{null}$  and  $V_{command}$ , and is subtracted from the output at a later stage.

level. The experimenter needs to take care to avoid damage to these from static potentials, for instance by simultaneously touching an earth when connecting a pipette.

(ii) The *feedback resistor* determines the sensitivity, the background noise level and range of current measurement. If  $i_p$  is the patch membrane current and ( $V_{out} - V_{ref}$ ) the voltage output,

$$-i_p R_f = V_{out} - V_{ref}$$

The feedback resistor contributes current noise which decreases inversely with the resistance. As described above, the variance of current noise at bandwidth  $f_c$  is given by

$$s^2 = 4kTf_c/R_f$$

It follows that for low noise, high gain recording,  $R_f$  should be high, say 50 G $\Omega$ .

However, since  $V_{out}$  will swing by a maximum of only  $\pm 12$  V, the output of the headstage will be saturated if  $i_p$  exceeds 240 pA. In whole-cell recording, currents may often exceed this level. The value of  $R_f$  must therefore be chosen to suit the experiment, either by prior selection of a suitable headstage or by using an amplifier that enables the experimenter to switch the value of  $R_f$  remotely. Several commercial instruments possess the latter facility. Alternatively, a good compromise may be achieved with a fixed 5 or 10 G $\Omega$  resistor for  $R_f$ .

*Capacitor feedback.* The noise associated with the feedback resistor is eliminated by using instead a capacitor to feedback current to the inverting input, producing an output that is the integral over time of the pipette current. To correct for this the output is taken via a differentiator stage. The improvement in signal to noise is about

30% when noise from other sources is minimised, a worthwhile improvement for high resolution single channel recording. The disadvantage is the need to discharge the voltage on the capacitor as the output voltage limit of the amplifier is approached, producing a reset transient which, although brief (50  $\mu$ s) may occur frequently with large standing currents such as those encountered in whole-cell recording.

(iii) The potential in the pipette is equal to  $V_{\text{ref}}$ . This potential is set for zero current by offsetting electrode potentials at the beginning of the experiment. This can be done either manually or by using the 'search mode' or 'tracking mode' of the patch clamp amplifier, which uses an integrator to keep the current at zero, adjusting  $V_{\text{ref}}$  accordingly. Once a high resistance seal is obtained, and the amplifier is switched to its voltage clamp mode,  $V_{\text{ref}}$  may be changed without causing large currents between pipette and bath, (such as those that occur with the 'loose-patch' method). Changing  $V_{\text{ref}}$  will change the patch membrane potential. Most patch amplifiers possess a 10-turn potentiometer, labelled  $V_{\text{HOLD}}$  or  $V_{\text{PIP}}$ , which allows the holding potential of the patch to be altered. Pulses from an external source ( $V_{\text{COMMAND}}$ ) may be used to change  $V_{\text{ref}}$  in a step-wise way. Generally, since noise applied to the headstage with the command signal appears in the current trace, the command voltage (and the noise applied with it) is divided 10- or 50-fold in the headstage, requiring a command pulse 10- or 50-fold larger than the pipette potential but with better signal/noise.

(iv) Fast changing commands, such as the leading edges of rectangular pulses, give rise to large currents due to charging stray capacitance associated with pipette and cell. These may saturate the amplifier and must be offset by adjustment of compensation circuits. Separate compensation is usually provided for 'fast' (primarily stray) and 'slow' (cell) capacitance.

(v) Particular attention needs to be paid to earthing and screening. The signal earth point of the amplifier should attach directly to the principal earth point of the set-up. The earth (or ground) socket on the headstage is connected within the cable to the signal earth in the patch clamp amplifier and is for connexion of the bath electrode. All metal surrounding the bath (baseplate, manipulators, dish holders, etc) and the microscope stage, nosepiece, objectives and condenser should have low resistance ( $<0.5\Omega$ ) connexions to the principal earth point. The case of the headstage amplifier may be connected to  $V_{\text{ref}}$  to reduce charging transients during voltage steps. The case should therefore be isolated from earth when it is mounted on the micromanipulator.  $V_{\text{ref}}$  may be available at a socket on the headstage with the intention that it might be used to minimise capacity current transients by connection to screens near the pipette (a 'guard' potential).

(vi) It is important to remember the convention for membrane current, viz. outward flux of cations (or inward flux of anions) is positive. The output polarity of a patch clamp may be + or -  $R_f i_p$  as determined by the internal circuitry after the headstage and therefore may be correct for whole-cell recording (and inverted for cell-attached), as is the case in most commercial instruments, or may be correct for cell-attached and incorrect for whole-cell recording (e.g. the Biologic amplifier has this arrangement).

(vii) The following instruments are particularly useful for patch clamp work: a 4- or 8-pole lowpass filter, normally with a Bessel characteristic; an FM or digital (VTR

or DAT) tape recorder; a computer interface and software for on-line voltage pulses and data analysis, and a wide bandwidth chart recorder such as a UV, light pen (Medelec) or electrostatic type.

## 4. Patch pipettes

### *Choice of glass*

The very high resistance of the seal between cell membrane and glass pipette means that some hydrophobic chemical interaction occurs between the two. The chemical composition of the glass may therefore influence the ability to form seals, although the comprehensive review by Rae & Levis (1992) of the properties of glasses of different composition indicates that no firm conclusions can be drawn concerning the ability of different types of glass to form seals. Other considerations that need to be taken into account in choosing electrode glass are as follows:

(i) The ability to form pipettes whose tips have an appropriate size and taper, factors that may influence the area of membrane isolated in a patch or the series resistance in whole-cell recording,

(ii) The degree to which background noise needs to be reduced.

(iii) Glass is doped with heavy metals as minor components to reduce the melting point. Heavy metal ions may leach into the pipette solution and modify channel properties.

The composition and properties of different types of glass are discussed by Rae & Levis (1992).

The following types of glass are commonly used.

*Soft (low melting point) glass.* This type of glass was initially used for patch clamp recording because it is easily pulled and shaped at relatively low temperatures, producing large aperture pipettes with blunt tips. It is not so commonly used to make recording pipettes but can be useful for making large aperture tips for cleaning tissue slices or drug application. Some soft glasses contain lead or barium, and haematocrit tubes are often made of this type of glass.

*'Pyrex' borosilicate glass (Corning type 7740).* Standard microelectrode glass is usually of this type, which is also most commonly used for patch pipettes. Pulling and polishing require high temperatures, usually orange heat of a platinum wire. Some argue that the sealing properties are better than those for soft glass (see Sakmann & Neher, 1983) although Hamill *et al.* (1981) state that seals are less stable with glass of this type. Low resistance pipettes (tip diameter 1-3  $\mu\text{m}$ ; resistance 1-5  $\text{M}\Omega$ ) are most easily pulled from thin walled glass. Thick walled pipettes generally seal better and have lower noise levels, because of the lower electrode capacitance (see below). The facility to back-fill pipettes made from electrode glass that has a fused internal filament is particularly convenient.

*Glasses for low-noise single channel recording.* The current noise in high resolution recording is influenced by the type of glass used because of differences in the resistive and capacitative properties of the thin glass wall at the tip. These

properties are reviewed by Rae & Levis (1992). The most significant points noted are (i) the large improvement in noise levels produced by Sylgard or other coating of the pipette shank with all types of glass and (ii) the considerably superior properties of quartz pipettes conferred by the low leakage conductance across the glass at the tip. Quartz softens at temperatures too high for conventional electrically heated filaments and pipettes are pulled either with a gas/oxygen flame or by a laser heated puller.

*Effects of glass on channel properties.* Two studies show that the type of glass used to make pipettes can affect the properties of channel recordings. Cota & Armstrong (1988) showed that two types of low melting point glasses soda or potash glasses produced additional fast inactivation of K channel currents when compared with channel properties determined with a borosilicate glass. This was subsequently shown to be due most likely to Ba ion release from the soda glass (Copello *et al.* 1991). Furman & Tanaka (1988) compared the properties of cGMP-activated channels in photoreceptor membrane recorded with pipettes made from one of six types of glass. They found differences in the current/voltage relation that could be reversed when EDTA or EGTA (which bind heavy metals with high affinity) were included at mM concentration in the pipette solution with all but one of the glasses tested. These and other reports indicate the necessity to avoid those types of glass that have been shown to affect channel properties and to test for contamination with chelators of heavy metals and by comparing different glass types before use on channels of well defined properties.

A second potential source of contamination should be noted. This arises from the use of stainless steel needles for backfilling pipettes, which are thought to be a source of polyvalent ions. It has also been reported that the hardeners in some plastic syringes can block nicotinic receptor/channels.

#### *Manufacture and fire polishing of pipettes*

Fig. 4 illustrates the double pull procedure used to make patch pipettes. With a conventional upright puller, the solenoid is disconnected and gravity alone provides the pull. Consistency in pipette formation can be increased if a digital voltmeter (set to  $V_{\text{rms}}$ ) is used to monitor the voltage across the heating element.

Fire polishing of pipettes is done with a heated Pt wire, mounted on the stage of a compound microscope, with stage movements or micromanipulators used to bring pipette tip and wire close together. High magnification (200-400 $\times$ ) good quality optics makes this step easier to carry out.

The tip diameter of pipettes may be estimated before and after fire polishing by measuring the pressure needed to expel air bubbles from the tip into clean methanol. This pressure may be measured as the volume change applied to a 10 ml syringe connected to the pipette. As a guide, starting at 10 and using the scale on the syringe, a bubble number of 5 or less indicates pipettes of  $>10 \text{ M}\Omega$  resistance suitable for cell-attached recording, of 6 or more low resistance pipettes suitable for whole cell recording. This procedure is useful particularly when setting up a pipette puller.

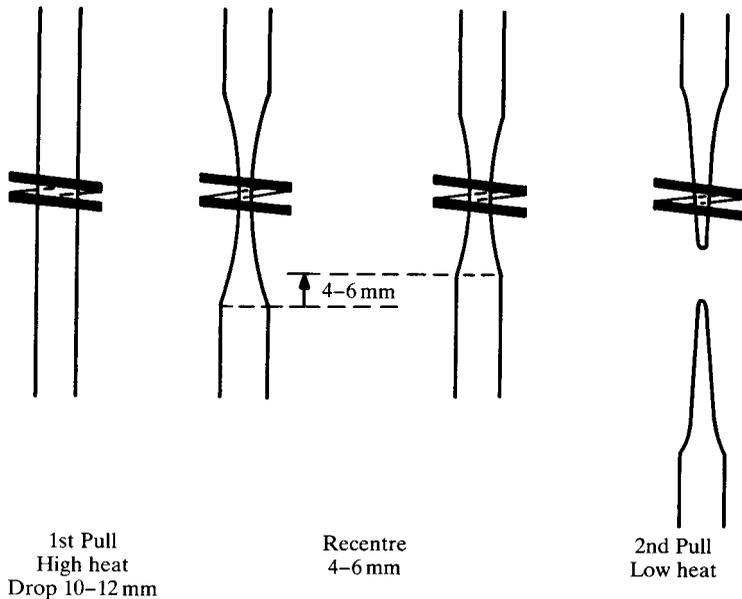


Fig. 4. Two-stage pull of patch pipettes.

#### *Coating pipettes to reduce capacitance*

Pipettes used in single channel recording are usually coated to thicken their walls and reduce capacitance to the bath solution. There are two reasons for wishing to minimize this stray capacitance. First, high frequency noise levels are reduced and secondly, as mentioned above, capacity currents associated with stepping the potential of the inside of the pipette are also reduced.

The r.m.s. current noise associated with stray input capacitance is given by

$$s_i = 2\pi f_c \cdot C \cdot s_v$$

where  $s$  is the r.m.s. noise for current ( $i$ ) and voltage ( $V$ ),  $f_c$  is the bandwidth, and  $C$  the total capacitance from input to earth. Current noise increases in proportion with voltage noise as the recording bandwidth is increased and also with the capacitance.

The current needed to charge the stray capacitance on changing the pipette potential is given by

$$i = C \cdot dV/dt,$$

and will be particularly large for a fast potential step. If at the leading or trailing edge of such a step  $dV/dt = 100 \text{ mV}/20 \mu\text{s}$ , the current needed to charge a stray capacity of  $10 \text{ pF}$  will be  $50 \text{ nA}$ . Since a headstage with a  $50 \text{ G}\Omega$  feedback resistor will be saturated by a current that exceeds  $0.24 \text{ nA}$  (see above), saturation would occur and important information about channels that are rapidly activated by the voltage step would be lost. Generally the *fast* capacity compensation circuits of patch amplifiers have a range too small to compensate fully pipette capacities larger than  $10 \text{ pF}$ .

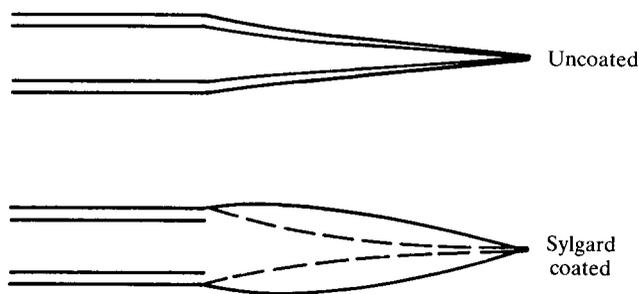


Fig. 5. Patch pipette before and after coating with Sylgard and curing.

Most of the stray pipette capacitance arises across the pipette wall between the pipette and the bath solution. It can be reduced by coating the pipette with a thick layer of Sylgard 184, an inert, hydrophobic, translucent elastomer resin rapidly cured by heating. The coat both thickens the wall of the pipette and reduces the area of capacitance coupling by preventing the bath solution creeping up and wetting the pipette. Coating should run from close to the tip (preferably closer than  $250\ \mu\text{m}$ ) and continue up the pipette well past the beginning of its taper. The procedure for coating pipettes and curing the Sylgard is illustrated in Fig. 5. As an alternative to Sylgard coating, pipettes can be dipped with backpressure before filling into a molten mix of 'Parafilm' in mineral oil (or some other molten wax) or after filling immersed in a silicone coating such as 'Sigmacote'. Both these procedures prevent fluid contact up the shank of the pipette and thicken the wall at the tip.

It is best to observe two additional measures to reduce stray capacity. First, the depth of bath solution in contact with the pipette wall should be minimized. Secondly, the pipette holder should be kept free of excess fluid; often it is helpful to dry the holder with compressed air or  $\text{N}_2$  before inserting a new pipette. A discussion of methods for low-noise recording is given by Rae & Levis (1992).

## 5. Formation of a gigaseal

As the pipette is advanced through the surface of the bath solution, slight positive pressure is applied to the inside of the pipette to keep the tip free of contamination, either by mouth or from a manometer (a water-filled U-tube that permits fine pressure adjustment by changing the pressure head of water). The current is set to zero, either by altering  $V_{\text{ref}}$  manually with the  $V_{\text{null}}$  control or by using the search mode of the amplifier. The pipette resistance is then monitored by measuring the current in response to 0.1 to 0.5 mV step-changes in  $V_{\text{ref}}$ . Contact with the cell is indicated by a rise in pipette resistance. *Gentle* suction (5-20 cm of water) is now applied to the pipette, when a seal should be formed. Seal formation may occur immediately, or it may take some while to happen. It may be helped by polarisation of the pipette to around  $-40\ \text{mV}$  once a high resistance has developed.

A fresh pipette should be used for each attempt to make a seal.

The process of seal formation can be observed with good microscope optics. Gigaseals may form immediately on contact with the cell surface with very little negative pressure, particularly with high resistance pipettes. In these cases the profile of the membrane is flat within the pipette tip. More often an  $\Omega$ -shaped region of membrane is drawn into the pipette tip and the seal forms initially slowly and suddenly achieves a high value. Photographs are given of membrane-pipette seals by Sakmann & Neher (1983). Milton & Caldwell (1990) report the formation of a bleb of membrane lipid in the pipette tip based on microscope observations of seal formation and propose a role for the bleb in forming gigaseals. Sokabe & Sachs (1990) give a microscopic analysis of the pressure/area relations of membrane patches.

#### *Giant patches*

Hilgemann (1989, 1990, see Collins *et al.* 1992) reports the use of various hydrophobic mixtures to facilitate the formation of seals with large tipped pipettes. These include viscous Parafilm/oil mixtures and  $\alpha$ -tocopherol. With minimal suction, these agents promote the formation of flat stable seals desirable for access to the inner surface of inside-out patches.

#### *Liquid junction potentials*

Where differences in ionic composition exist between pipette and bath solution, a correction must be applied for the junction potential across the boundary between the two solutions. This potential arises from differences in concentration and mobility of the ion species in the two solutions and maintains electroneutrality across the boundary between the two solutions. The magnitude depends on the relative mobility and concentration gradient of ions diffusing in the two directions and, depending on the solutions used, may be 2-12 mV in physiological solutions. The error arises because the potential will be present when pipette current is zeroed before sealing, but when the seal is made the concentration gradients form part of the driving potential for ion movement, leaving the applied offset potential still imposed in the pipette.

With reference to Fig. 3, the initial sequence of steps made during patch recording are as follows. (i) Zero current output of the amplifier is checked with the pipette out of the bath - this is usually zero volts. (ii) The pipette is put in the bath solution and an offset current flows as a result of asymmetries in the AgCl half cell potentials, the junction potentials across the pipette tip to bath and bath into the reference electrode salt bridge, and any voltage on the amplifier  $V_{\text{ref}}$ . For present purposes, suppose that the AgCl potentials are equal and opposite and the reference junction potential is zero, and so do not contribute. The current flowing into the pipette is driven by the difference between bath potential and pipette potential and is set to zero by adjusting  $V_{\text{ref}}$  (which sets the pipette potential with respect to the bath). In most patch amplifiers, this is done with a 'Null' potential control that changes  $V_{\text{ref}}$  without registering on the voltage monitor. This value of  $V_{\text{ref}}$  is taken as zero holding potential and is the zero current potential of the pipette with respect

to bath potential. However, by convention the polarity of the junction potential is bath minus pipette, opposite to the polarity of the  $V_{\text{ref}}$  potential applied in the pipette. (iii) A seal is formed and the liquid junction potential disappears, leaving the pipette potential displaced from true zero by an amount equal to the initial pipette-bath potential difference, opposite in polarity to the junction potential. In a cell-attached or inside-out recording, the potential is on the outside of the membrane and the true transpatch potential is the holding potential minus the initial pipette-bath potential (i.e. plus the conventional junction potential). For whole-cell recording on breaking through, a new junction potential appears between the pipette and cytosol immediately on patch rupture and disappears over the first minute or so as the cell is dialysed with pipette solution, leaving the initial pipette-bath potential added to the holding potential (i.e. the conventional junction potential should be subtracted).

The error due to the junction potential can be demonstrated and quantified fairly easily. However, it is necessary to have a reference junction that remains at zero when the composition of the bath is changed. This can be done with a 2-3 M KCl solution in the reference salt bridge and stable AgCl wire or pellet provided the junction between salt bridge and bath is continuously renewed by slow outflow of KCl; for example from a blunt pipette some distance from the recording site (see Neher, 1992; also Chapter 1). Zero junction potential is set at the recording pipette by having the same solution in the bath as would be in the pipette during an experiment. The potential is measured at zero current, either in voltage clamp or current clamp, and set to zero. The bath is changed to another solution (e.g. external solution) and the change in zero current potential measured. This is the excess potential that would be present in the pipette after seal formation and should be subtracted from the holding potential in cell attached or added in whole cell to give the real membrane potential.

This problem is reviewed, with particular reference to patch clamp recording, by Barry & Lynch (1991) and by Neher (1992). The polarity has been given the convention bath-pipette, opposite to that determined in the experiment above. The junction potential can be calculated from the Henderson equation (given e.g. in Barry & Lynch, 1991) if the activities and mobilities of the ions are known. As an example, in the simple but common case where the bath contains a 150 mM NaCl solution, while the pipette has an equal concentration of KCl, the junction potential can be predicted from:

$$E_{j.p.} = (RT/F) \cdot \ln[(u_K + u_{Cl})/(u_{Na} + u_{Cl})]$$

where  $u_{Na}$ ,  $u_K$ , and  $u_{Cl}$  are the mobilities of  $Na^+$ ,  $K^+$ , and  $Cl^-$  and  $E_{j.p.}$  is the junction potential, bath relative to pipette.  $E_{j.p.}$  has in this case a calculated value of +4.3 mV, so the pipette will have an excess potential of -4.3 mV with respect to the bath. In whole-cell recording, zero mV imposed in the pipette will be -4.3 mV; in cell-attached recording zero will be +4.3 mV. In cases where solutions differ more, particularly with  $Cl^-$  ion replacement, the junction potential can be calculated with equations and ion mobilities given by Barry & Lynch (1991) or taken from the measured values given by Neher (1992).

Further problems arise when junction potentials occur at the reference/bath solution junction. It is usual to have a salt bridge of NaCl bath solution between the reference AgCl electrode and bath. This has no junction potential into the bath unless the external solution is changed, when a potential may arise and add to the holding potential. Procedures for measuring liquid junction potentials and discussion of more complex effects seen with external cell perfusion by dissimilar solutions are given by Neher (1992).

A second, but less common problem, is that salts of low solubility product (e.g. calcium or barium phosphates, fluoride or sulphate) may precipitate at the interface of pipette and bath solutions.

## 6. Choice of preparation

The choice of cell to use for single channel work depends ideally on the experiment that is envisaged. In practice, the choice of preparation can hinge on whether its cells readily make seals with glass and on the density of ionic channels in the membrane. Preparations that are used include cells from adult tissues, cultured cells, channels in membranes of native lipid, and channels reincorporated into artificial bilayers as large vesicles.

### *Adult cells*

These have to be cleaned of connective tissue and extracellular matrix, using enzymes such as collagenase and proteases. When dissociating tissues, the presence of DNase helps prevent clumping; however, individual protocols for enzymatic dispersal are empirically determined. In CNS tissue slices, the cell surface can be cleaned by irrigation from a 'cleaning' pipette, and this procedure is increasingly applied to other tissues.

The channel density is often very high in adult tissue - sodium channels may have a density of  $100 \mu\text{m}^{-2}$  or more - making it impossible to isolate a single or small number of channels.

### *Cultured cells*

These provide the most widely used preparation for single channel recording. Channel density is often lower than in adult cells, and the membrane may have less extracellular matrix. Commonly used *primary cultures* include myotubes and explant neuronal cultures grown from new-born rats or mice, from *Xenopus* larvae and from invertebrates. Various cell lines which provide convenient models for physiological processes are also widely used.

### *Channels in native lipid membranes*

Large liposomes or vesicles have been developed using freeze/thawing of

microsomes (Tank, Miller & Webb, 1982) dehydration/rehydration procedures (Keller & Hedrich, 1992) or KCl and water loading of cells, coupled with protease treatment (Standen, Stanfield, Ward & Wilson, 1984).

### *Reincorporation*

Lipid membranes can be formed on the tips of patch pipettes and channel reincorporated into these bilayers from microsomes (Coronado & Latorre, 1983 see Chapter 5). Such a method is particularly useful in the investigation of channels in specific membrane fractions. For example, this and related methods can be used to investigate channels in membranes of the transverse tubular system of muscle fibres or in intracellular membrane systems such as sarcoplasmic reticulum and endoplasmic reticulum.

## **7. Cell-attached recording**

This mode of recording is used to study single channel currents. The resolution of such unitary currents should be optimized by observing the precautions detailed above for reducing stray capacitance. Good mechanical stability and freedom from vibration are needed to keep cell-attached patches for long enough to record sufficient data.

Activation of channels operated by binding of transmitters may be produced by having a suitable concentration of transmitter or agonist in the patch pipette. Opening of voltage-activated channels may be achieved by stepping the potential inside the pipette. Often the pipette solution will have the same ionic composition as the bath solution; substitution of ions may be made for studies of permeability properties of channels.

The membrane potential of cell-attached patches ( $V_{\text{patch}}$ ) is determined by that of the cell interior ( $V_{\text{cell}}$ ) as well as by the pipette ( $V_{\text{ref}}$ ). The membrane potential is therefore:

$$V_{\text{patch}} = V_{\text{cell}} - V_{\text{ref}}$$

Generally,  $V_{\text{cell}}$  is not known. Sometimes, for example in large cells such as skeletal muscle fibres, it is possible to record this potential with an impaling microelectrode. Suitable analogue means may be used to subtract it from  $V_{\text{ref}}$ , so that  $V_{\text{patch}}$  is the quantity monitored. Breaking the membrane patch at the end of the experiment with strong suction, so that the pipette is in contact with the cell interior, can also be used to obtain an indication of  $V_{\text{cell}}$ .

Examples of currents through nicotinic receptors, activated by acetylcholine, are shown in Fig. 6. Fig. 7 shows unitary potassium currents through delayed rectifier channels of skeletal muscle, activated by a depolarizing step. In these records, outward membrane currents are shown as upward deflections since they are, by convention, positive quantities. Most commercial patch clamp amplifiers are

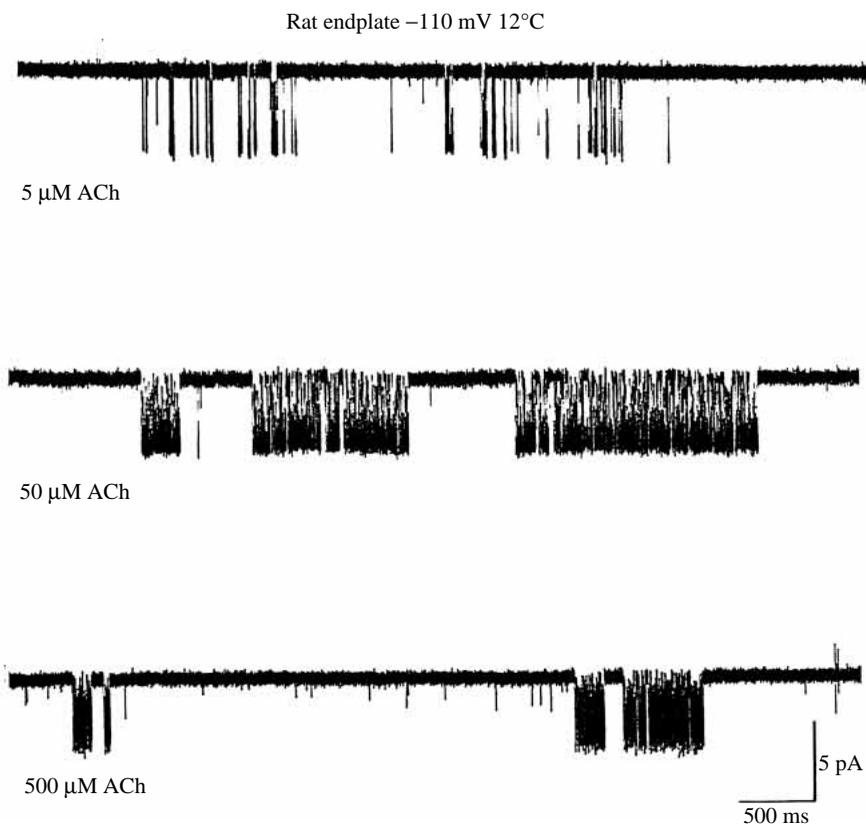


Fig. 6. Cell-attached recording of inward single channel currents activated by acetylcholine in the pipette solution at the rat skeletal neuromuscular junction. Potential  $-110$  mV.  $12^{\circ}\text{C}$ . Bandwidth 4 kHz. Note the grouping of channel openings into bursts and clusters and the increase in open probability within clusters as the ACh concentration was increased. N. K. Mulrine and D. Ogden.

arranged so that current flowing into the pipette (outwards across the membrane of cell-attached patches) produces a negative output. For clarity in description of their experiments, patch clampers should be careful to use the correct convention for membrane currents and membrane potentials.

*Recording from expressed channels.* Ion channels modified by molecular biological procedures and expressed in cell membranes are used to study the relation between structure and function. They can be transfected in cell lines or expressed in *Xenopus* oocytes by injection of mRNA into the cytosol, or of DNA into the nucleus. Procedures for recording from cell lines are the same as those described above. Recording from oocytes is technically more difficult because of the presence of accessory follicular cells and a vitelline membrane which must be removed to gain access to the plasmalemma. Procedures for treating oocytes for patch recording are described by Methfessel *et al.* (1986).

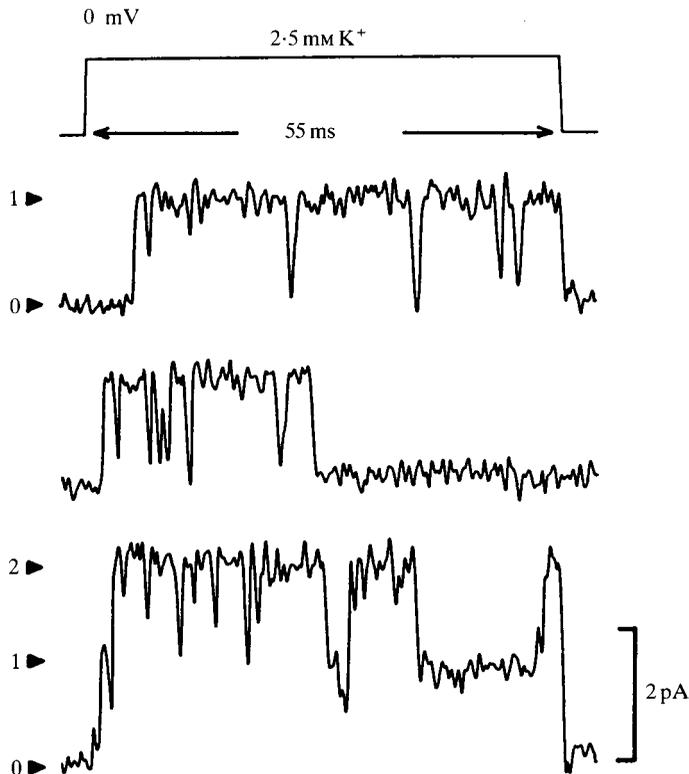


Fig. 7. Single channel records of potassium channel currents in skeletal muscle fibre activated by a 55 ms depolarization of the membrane potential from  $-100$  mV to  $0$  mV. Cell attached recording. Note three levels of current corresponding to simultaneous opening of 0, 1 and 2 channels. Reproduced from Standen, Stanfield & Ward (1985), with permission.

## 8. Cell-free, excised patches

Inside-out patches are made from the cell-attached configuration as indicated in Fig. 2. Pulling off a membrane patch often results initially in the formation of a vesicle of membrane in the pipette tip. The outer face must be broken open, which may be done by briefly taking the membrane through the bath solution/air interface; by exposure to a low  $\text{Ca}^{2+}$  solution; or by momentarily making contact with a droplet of paraffin or a piece of cured Sylgard.

Once the patch is formed, channels activated or modulated by substances applied to the cytoplasmic membrane face may be studied. Such studies have included investigations of the effects of  $\text{Ca}^{2+}$  (Marty, 1981; Barrett, Magleby & Pallotta, 1981; Colquhoun, Neher, Reuter & Stevens, 1981); of catalytic subunit of protein kinase (Shuster, Camardo, Siegelbaum & Kandel, 1985); and of ATP (Noma, 1983; Spruce, Standen & Stanfield, 1985).

Outside-out patches are formed after breaking into the cell with the procedure

indicated in Fig. 2. Such patches have been used by a number of workers for the study of the pharmacology of channels activated by transmitters or hormones (Hamill, Bormann & Sakmann, 1983; Gardner, Ogden & Colquhoun, 1984; Nowak, Bregestovski, Ascher, Herbert & Prochiantz, 1984; Cull-Candy & Ogden, 1985). This configuration of the cell-free mode often has the disadvantage of a higher level of background noise, probably because of a lower resistance of the seals achieved and because of the larger area of membrane in the patch. Further it has been reported that channel kinetics may differ when recorded in outside-out, excised patches from those found in cell-attached recording (Trautmann & Siegelbaum, 1983; Fernandez, Fox & Krasne, 1984).

*Fast concentration changes.* To study neurotransmitter-activated channels, it is important to be able to apply the neurotransmitter on a timescale similar to that encountered in the synapse, within 1 ms. This can be achieved with outside-out patches because of the very narrow unstirred layer of solution adjacent to a patch when compared to that surrounding a whole cell, where solution changes require tens of milliseconds. Methods for fast, submillisecond, concentration changes have been described by Maconochie & Knight (1989) and Lui & Dilger (1991) utilising electronic valves, and with piezo driven stepping of a stream of solution onto the pipette tip by Dudel, Franke & Hatte (1990).

## 9. Whole-cell recording

Whole-cell recording is achieved by rupturing the patch of membrane isolated by the patch pipette, which brings the cell interior into contact with the pipette interior. The process of whole-cell recording is as follows.

After forming a gigaseal, the fast capacity transients associated mainly with pipette capacitance to the bath are compensated. Next, the potential of the pipette interior ( $V_p$ ) is adjusted to a level similar to the anticipated membrane potential of the cell. Rupture of the membrane patch is achieved by applying strong suction or sometimes brief voltage transients, and the process is conveniently monitored by applying test pulses of 1 to 5 mV amplitude. Rupture is indicated by the sudden appearance of large capacity transients at the leading and trailing edges of the pulse. The sequence of changes is shown in Fig. 8.

### *Series resistance errors in whole cell recording*

The equivalent circuit used to represent the whole-cell recording condition is illustrated in Fig. 9. Current in the pipette, defined for whole-cell recording as positive flowing from pipette to cell, flows first through a resistance in series with the cell membrane to represent the pipette tip. If this series resistance is  $R_s$  and the pipette current is  $i_p$ , a potential difference pipette – cell of amplitude  $i_p R_s$  results. This potential needs to be subtracted from the command potential to yield the cell potential. The series resistance compensation present in commercial patch clamps allows a fraction of  $i_p R_f$  to be added to the command to partly correct the error.

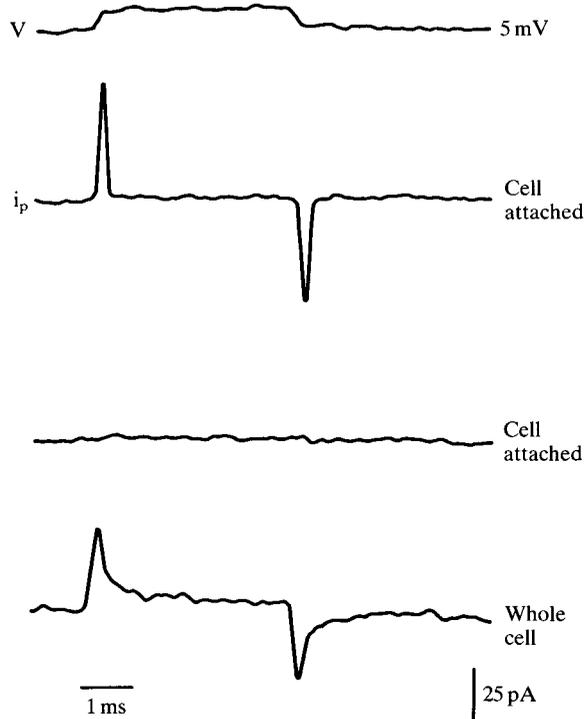


Fig. 8. (a,b) Transient currents due to stray (pipette) capacitance in a cell-attached record before (a) and after (b) cancellation with  $C_{fast}$  and  $\tau_{fast}$ . (c) Transients due to cell capacitance in a whole cell recording with 5 mV rectangular input to  $V_{ref}$ . 2 kHz bandwidth. The cell capacitance was 4 pF.

The pipette current has two components, one ionic ( $=V_c/R_c$ ) and the other capacitive ( $=C \cdot dV_c/dt$ ). The time course of the change in the potential of the cell,  $V_c(t)$ , from its initial value  $V_c(0)$  is given by:

$$V_c(t) = \{V_c(0) - V_c(\infty)\} \{1 - e^{-t/\tau}\}.$$

$V_c$  changes on an exponential curve of time constant

$$\tau = C \cdot R_s R_c / (R_s + R_c),$$

usually approximated well by  $\tau = C \cdot R_s$  because  $R_c \gg R_s$ .

This time constant characterizes the response of the voltage clamped cell not only to applied voltage steps, but also to currents originating in the cell membrane, which are therefore effectively low pass filtered with a bandwidth

$$f_c = 1/(2\pi\tau).$$

The time course of the current following a sudden change of  $V_p$ , also shown in Fig. 9, has a declining exponential timecourse. Initially current flows mainly into the capacitance. Since  $V_c(0)$  is steady at the beginning of the step in  $V_p$ , the initial pipette

current flowing into the cell discharges the capacitance and is given by  $i_p(0)=V_p/R_s$ . The pipette current declines exponentially with  $\tau=C.R_s$  to a steady level. The size and time constant of the error voltage due to series resistance is minimized simply by keeping  $R_s$  as small as possible by using low resistance pipettes. If the last condition is kept (and it may be expected to hold in most conditions),  $\tau=R_sC$  and for small currents  $V_c(\infty)=V_p$ .

When using whole-cell recording, it is important to know the errors that arise from series resistance and cell capacitance. These quantities may be estimated from the current transients shown in Figs 8 and 9. Provided  $R_s \ll R_c$ , the area of the transient, which gives the charge on  $C$ , is equal to  $C.V_p$ , while the time constant of the decline is given by  $\tau=R_sC$ . The area may be estimated by digitizing the current record and integrating, the time constant by curve fitting. Estimates of  $R_s = V_p/i_p(0)$  from the initial amplitude of the transient are subject to error because the rise of  $V_p$  usually has a time course of 10-20  $\mu\text{s}$  and because of low-pass filtering. Typical values of the series resistance are 3-20  $\text{M}\Omega$  if low resistance (1-5  $\text{M}\Omega$ ) pipettes are used. The resistance may be as high as 50  $\text{M}\Omega$ , however, and is liable to frequent fluctuation during recording. For a series resistance of 10  $\text{M}\Omega$  and a cell capacity of 12 pF (corresponding to a spherical cell of 20  $\mu\text{m}$  diameter of membrane capacitance 1  $\mu\text{F.cm}^{-2}$ ),  $\tau=120 \mu\text{s}$  for settling of the clamp and  $f_c=1.3 \text{ kHz}$ .

The currents associated with charging the cell capacitance should be compensated, using the 'slow' capacity compensation of the patch clamp amplifier. This system injects current into the pipette to cancel the capacitive currents, avoiding saturation of the headstage during large voltage pulses. In most patch clamp amplifiers, the slow capacity compensation is separately switched, and has controls for the size of the capacitance and the time constant (or series resistance) of the decline of capacity currents. It is important to note that capacity compensation subtracts capacity current from the amplifier output. It does not compensate for the slow change in cell membrane potential due to series resistance. Most commercial amplifiers have separate series resistance compensation which allows a fraction of the pipette current times series resistance to be added to the command voltage as in conventional voltage clamp (see Chapter on voltage clamp techniques). This works best if  $R_s$  is small and should be used with caution because of frequent fluctuations of  $R_s$  during recording, to the extent that the best procedure is to monitor and adjust the compensation digitally via an interface immediately before each voltage command. A second way of dealing with series resistance in whole-cell recording is to use a switched (discontinuous) single electrode voltage clamp in which the membrane potential is monitored at zero current flow, so eliminating the error voltage due to  $i_p$  (see Chapter 2). Switched clamps have inherently greater noise, 10-100 fold, than patch clamps and can only be used with large membrane currents.

#### *Diffusional exchange between pipette solution and cytosol*

In conventional whole-cell recording, the contents of the interior of the cell come into diffusional equilibrium with the solution in the patch pipette. It is achieved

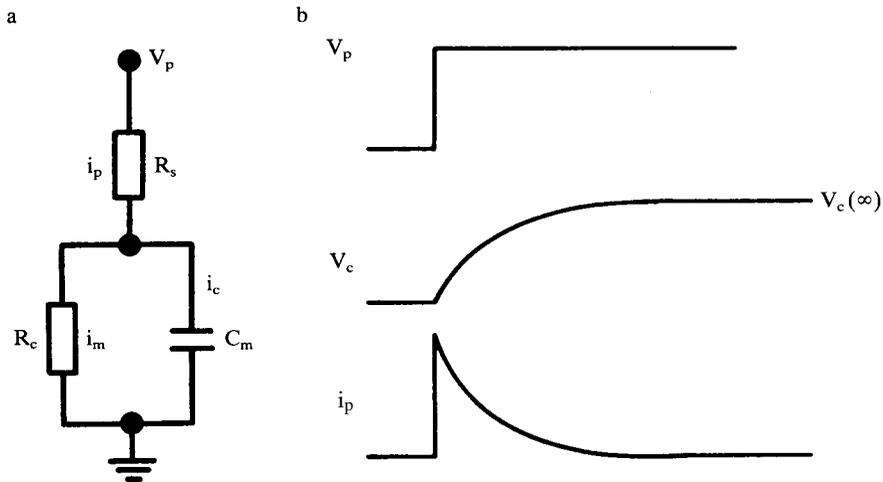


Fig. 9. (a) Equivalent circuit of whole cell recording. Current  $i_m$  flows in the cell resistance  $R_c$  and  $i_c$  in the capacitance. Pipette current,  $i_p = i_m + i_c$ , flows in the series resistance  $R_s$  between pipette and cell and produces a voltage error  $V_p - V_c = i_p R_s$ . (b) Time course of changes of  $V_c$  and  $i_p$  following a step of  $V_p$ .

$$V_c = V_p \frac{R_c}{R_c + R_s} (1 - e^{-t/\tau})$$

$$i_p = \frac{V_p}{R_c + R_s} + V_p \frac{R_c}{R_s(R_c + R_s)} e^{-t/\tau}$$

$$\tau = C_m \cdot R_s R_c / (R_s + R_c)$$

$$V_c(\infty) = V_p R_c / (R_c + R_s)$$

$$i_p(0) = V_p / R_s \quad i_p(\infty) = V_p / (R_c + R_s).$$

more rapidly for ions than for large intracellular molecules, which do, however, get washed out of the interior of the cell in time. The time course of exchange depends on the size of the pipette tip and the cell volume, as well as on the molecular size and the degree of buffering by cytoplasmic constituents. The advantages are control of the intracellular ionic composition and the perfusion into the cell of agents such as dyes that indicate ion concentrations, second messengers and 'caged' second messengers, antibodies to intracellular proteins and small proteins generally. The disadvantage is the loss of important cellular components, including small proteins, into the pipette.

The time course of equilibration of  $\text{Na}^+$  ions during whole-cell recording was reported by Fenwick, Marty & Neher (1982) to have a time constant of about 5 s in chromaffin cells, judged by changes in the Na current.

Junction potentials due to small ions will exist between pipette solutions and the cytoplasm. This source of error will not be a constant, but will change towards zero as equilibrium between pipette and cellular contents proceeds. Marty & Neher (1983)

estimate a maximum error of 12 mV from this source. A displacement of the potential dependence of ionic currents that occurs during whole-cell recording may be related to this changing junction potential.

The time course of equilibration of larger molecules is much slower, particularly if they bind to cellular components or are metabolised. For molecules such as fluorescent dyes of mass number 500-1000 Da equilibration, judged by the fluorescence increase, takes several minutes, and depends very much on how good the access is through the pipette tip. Observation suggests that as well as a low access resistance it is important to minimise the amount of material taken into the pipette tip during seal formation. An analysis of the time course of equilibration of dextran-tagged dyes as a function of size and access resistance has been made by Pusch & Neher (1988). They present evidence that components of upto about 20 kDa may be lost from the cell.

There are well documented instances of changes in the properties of ionic currents, and also cell responses involving second messengers, that occur during whole cell recording and which can be attributed to loss of cytosolic components into the pipette. Best known is the rundown of slowly-inactivating Ca conductance that occurs during intracellular perfusion. Evidence suggests that this is slowed by inclusion of ATP and Mg in the pipette, and may be improved by inclusion of protease inhibitors such as leupeptin or phosphatase inhibitors. Loss of the calcium mobilising hormonal response of exocrine cells during conventional whole cell recording was noted by Horn & Marty (1988) who introduced the method of *patch permeabilisation* to prevent rundown of the responses. Instead of rupture by pressure of the membrane under the pipette they achieved electrical access to control the membrane potential by including the Na-ionophore nystatin in the pipette solution, preventing loss of high molecular weight components. The method has been used with other ionophores, such as amphotericin, and a bacterial toxin ( $\alpha$ -toxin from *Staph. aureus*, Khodakhah *et al.* 1992) that makes pores large enough to admit dyes and 'caged' second messengers <1 kDa. Permeabilisation is generally more difficult to implement than patch rupture because the ionophores prevent seal formation and are unstable in solution.

Use has also been made of the exchange of cell and pipette constituents during whole cell recording to change the concentrations of internal ions and of other substances in a systematic way. A perfusion system that takes account of the technical problems encountered has been described by Soejima & Noma (1984).

#### *Measurement of changes in membrane capacitance*

The facility of whole cell recording to make precise measurements of membrane capacitance has been developed as a method for measuring changes in membrane area that occur during exocytosis. The procedure is to analyse the admittance of the whole cell equivalent circuit into components due to capacitance, series resistance and membrane conductance by use of a phase-locking amplifier. The resolution is sufficient to see unitary changes associated with the incorporation of single (albeit 'giant') vesicles into the cell membrane, and has given considerable information

about the process of vesicle-membrane fusion (reviewed by Almers & Tse, 1990). Technical descriptions of the method are contained in the initial report by Neher & Marty (1982) and in articles by Lindau & Neher (1988) and Fidler & Fernandez (1989).

## 10. Identification of ionic channels

The nature of ionic channels may be identified by (1) the mode of activation; (2) the permeant ions (under physiological conditions); (3) the unitary conductance; and (4) selective block by drugs, ions or toxins.

(1) Ionic channels may be opened by the following. (a) Neurotransmitters and chemical analogues acting on external receptors coupled directly to the channel opening mechanism. The open probability of these channels depends on transmitter concentration and they are usually involved in fast synaptic transmission. Examples are nicotinic acetylcholine channels and glutamate-activated channels. (b) Membrane potential, for example sodium, potassium and calcium channels opened by depolarisation during the action potential. (c) Intracellular ion concentration (e.g. Ca-activated K-channels) or other cellular constituents (e.g. K-channels closed by internal ATP). (d) The open probability of some channels is modified by intracellular second messengers released by hormone action. An example is the Ca channel of cardiac muscle, after phosphorylation by cAMP-dependent protein kinase. Most of these kinds of channels are also opened by one of the above means.

(2) Once a channel is opened, the ions that permeate can be determined by measuring changes of the reversal potential and unitary conductance when the concentration of ions in the external or internal medium is changed.

(3) The unitary conductance under specified conditions may vary considerably among different channels with the same ion selectivity, for example there are at least 3 types of Ca-activated K-channel distinguishable on this basis.

(4) Some channels are blocked selectively by low concentrations of drugs or toxins, for example block of Na channels by external tetrodotoxin, of delayed rectifier K-channels by TEA derivatives or of nicotinic acetylcholine channel activation at the neuromuscular junction by  $\alpha$ -bungarotoxin. Block by other agents may be less selective; for example block of Na or acetylcholine channels by local anaesthetics or K-channels by quinine.

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